To: Defense Technical Info Ctr.
8725 John M Kingman Rd., Suite 0944
Fort Belvoir, VA 22060-6218

RE: ONR Award: N00014-11-1-0540 – "Assessing Stress Responses in Beaked and Sperm Whales in the Bahamas"

Please find attached final reports for the above referenced ONR award for the period ending March 31, 2016. *(Please confirm via email that you have successfully received these final reports).*

Attachments include:

1.) Final Technical Report (MBrollan.docx)
2.) Rolland_SF298: Federal from required by ONR (not sure if 298 is required, please disregard if not.)

If you have questions or concerns contact my office.

Best,

-S

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Assessing Stress Responses in Beaked and Sperm Whales in the Bahamas

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http://tinyurl.com/MarineStress
http://www.bahamaswhales.org

LONG-TERM GOALS

The long-term goal of this project was to develop fecal hormone assays to assess stress responses in Blainville's beaked whales (*Mesoplodon densirostris*) and sperm whales (*Physeter macrocephalus*) inhabiting the northern Bahamas. These deep-diving species were chosen to investigate a particularly acoustically-sensitive cetacean (beaked whales) and a co-occurring species (sperm whales) for comparison. The physiologic data generated by this project will provide baseline levels of stress and metabolic hormones in free-swimming beaked and sperm whales for comparison with conspecifics experiencing known acoustic disturbances (including mid-frequency active sonar), such as at the nearby U.S. Navy Atlantic Undersea Test and Evaluation Center (AUTEC).

OBJECTIVES

(1) Conduct dedicated fecal sampling surveys for reference populations of Blainville's beaked whales and sperm whales off southwest Great Abaco Island, refine methods to maximize sampling rates, and determine the feasibility of fecal sampling for hormone analyses in both species. Use photo-identification coupled with genetic analyses to provide information on the sampled individual's age, sex, and reproductive state.

(2) Validate immunoassays for fecal reproductive hormone metabolites (estrogens, progestins, androgens), adrenal "stress" hormones (glucocorticoids, GCs) and thyroid hormone (T3) for beaked and sperm whales. Process and analyze all fecal samples for a panel of these five hormones.

(3) Characterize baseline levels and the natural variation of these five hormones according to life-history state (age, sex, reproductive state) in minimally disturbed Blainville's beaked whales and sperm whales.

APPROACH

This project was a collaboration between scientists at the John H. Prescott Marine Laboratory at the New England Aquarium (NEAq; Boston, MA) and the Bahamas Marine Mammal Research Organisation (BMMRO; Great Abaco Island, The Bahamas). Sample collection was led by BMMRO scientists (D. Claridge, C. Dunn) with assistance from NEAq scientists (R. Rolland, S. Kraus, K. Hunt, E. Burgess). Fecal samples were collected from Blainville's beaked whales and sperm whales off SW Great Abaco Island using methods previously employed by BMMRO with modifications made by NEAq scientists. Initial sample processing was conducted in the Bahamas in a field laboratory equipped with a centrifuge and basic equipment at BMMRO. BMMRO provided individual identification and life history information on sampled whales. Further sample processing, extractions, hormone validation studies, hormone assays, data interpretation, project oversight and management, and reporting were conducted by the NEAq (R. Rolland, K. Hunt, E. Burgess, K. Graham, S. Kraus).

WORK COMPLETED

Task 1. Field Effort in the Bahamas

Dedicated sampling surveys were conducted during 131 vessel-days over four years. This work was performed during June to September from 2011 to 2015 (excluding 2012) using 5.7m and 6.8m rigid-hulled inflatable boats. Surveys were primarily concentrated near the 1000m isobath along the southwest side of Great Abaco Island in the northern Bahamas (Fig. 1). The total effort constituted 5,158km of vessel track lines.

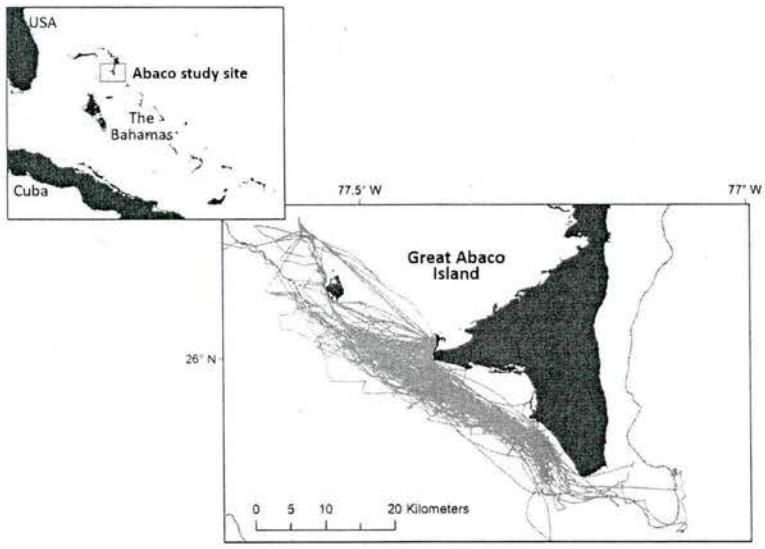

Figure 1. Map showing vessel tracks during dedicated and leveraged surveys off the coast of Great Abaco Island between 2011 and 2015. Survey efforts were concentrated off the southwestern side of the island along the 1000 m isobaths (black dashed line).

Fecal Sample Collection

Fecal samples were collected from free-ranging Blainville's beaked whales and sperm whales using methods previously employed by BMMRO with modifications made by NEAq scientists in FY2011 to maximize sample collection (Rolland et al. 2011). In FY2013 a field laboratory was set-up at BMMRO, including installation of a portable centrifuge for preliminary processing of samples (Rolland et al. 2013). Beaked whale feces were collected in the water column by a towed diver in snorkel gear using a small dip net and a one gallon plastic zip-type bag (Fig. 2). Sperm whale samples were scooped off the water surface into Falcon tubes or a plastic sample jar. Sample collection was accompanied by photo-identification (when possible), and images of sampled whales were compared to existing identification catalogues to provide information on the individual's age-class and sex. Samples were stored in a cooler with ice-packs while at sea, centrifuged at the BMMRO field station the same day to remove water from the fecal material, and stored in a freezer (-18°C) until overnight shipment to the NEAq in an insulated cooler on dry ice. Control water samples were also collected in the vicinity of whales to test for background immunoreactivity in seawater.

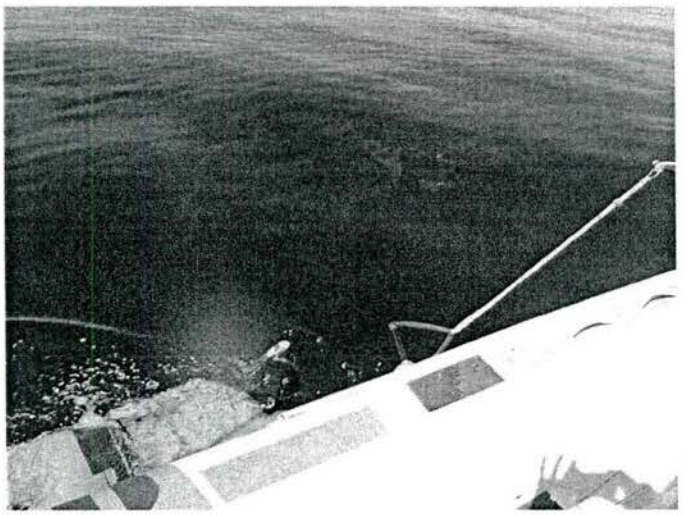

Figure 2. A diver in snorkel gear is towed alongside the research vessel to observe beaked whales underwater and collect fecal samples using a small dipnet when defecation is seen. Photo credit: BMMRO.

Task 2a. Hormone Assay Validations

Fecal sample processing and hormone extraction protocols were developed specifically for each species to maximize hormone yield (Rolland et al. 2012). Radio-immunoassays (RIA; 125I and 3H) for total-estrogens, progestins, androgens, glucocorticoids and thyroid hormone (T3) were validated for beaked and sperm whales using standard parallelism and accuracy studies (Grotjan & Keel 1996). In addition, we conducted validations on two enzyme immunoassays (EIA; estrone-1-glucuronide and testosterone).

Task 2b. Hormone Assays

Individual fecal samples were analyzed on five RIA systems, including progestins, androgens, total estrogens, glucocorticoids, and thyroid hormone (T3). Radio-immunoassay methods are detailed in Rolland et al. (2005, 2012), Hunt et al. (2006), and Wasser et al. (2010). All samples and standards were assayed in duplicate. Any sample with a coefficient of variation >10% between the duplicates was re-assayed. Samples showing percent bounds of < 10% or > 90% were re-diluted as needed, and

assayed again. Results are expressed as nanograms of immunoreactive hormone per gram of dried feces (ng/g).

Task 3. Data Analysis, Publications and Reporting

Data analyses have been completed and at least one scientific publication on this work is anticipated. Because of non-normal distributions, data were $\log_{10}$ transformed before most statistical analyses. Standard descriptive statistics were used to characterize hormone data. Analyses of hormone concentrations used Student's *t*-tests, One-way ANOVA with Tukey's HSD post-hoc tests or a Bonferroni correction for multiple comparisons, and Pearson's or Spearman's correlation coefficients. Results were considered significant at $p < 0.05$.

RESULTS

During surveys, there were 166 cetacean sightings including eight different species, which comprised 35 groups of Blainville's beaked whales and 33 groups of sperm whales. Group size ranged from 1-7 whales for Blainville's beaked whales (mean = 5) and 1-14 animals for sperm whales (mean = 5).

Fecal Sample Collection

Almost all fecal samples were collected off Great Abaco Island (Fig. 3). Five additional sperm whale samples were collected opportunistically in the vicinity of the AUTEC range offshore of Andros Island during the course of research conducted under other funding.

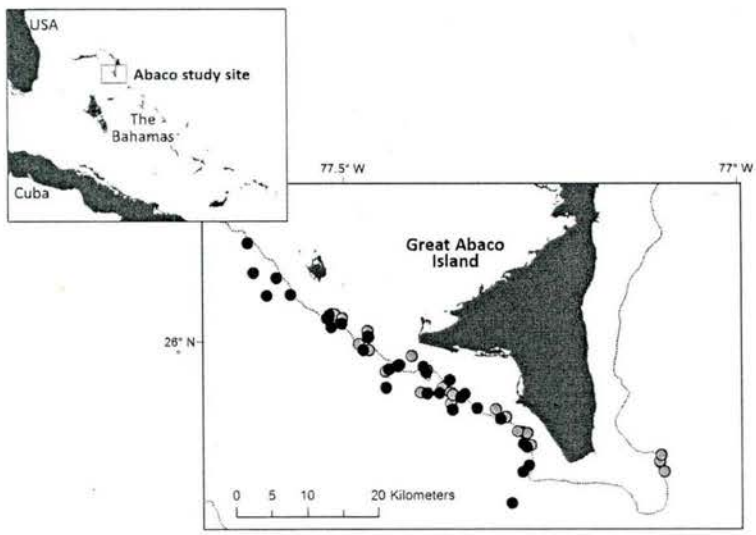

Figure 3. Map showing locations where fecal samples were collected during surveys off Great Abaco Island between 2011 and 2015. Blainville's beaked whale sample locations are represented by orange circles and sperm whales by black circles. The 1000 m isobath is shown by the gray dashed line.

Sixty-nine fecal samples were collected during the field effort from Blainville's beaked whales (n = 28) and sperm whales (n = 41) (including 8 sperm whale samples collected under other project funding at Abaco and AUTEC; Table 1). Up to five samples were collected from beaked whales in a single survey day. The success of beaked whale sampling, in particular, was dependent upon a Beaufort sea state ≤ 2 and good underwater visibility. Once a group of whales was located, for beaked whales the

average encounter time per sample collected was 99 min. (range: 50-138 min/sample), and for sperm whales it was 111 min. (range: 12-132 min/sample).

Table 1. Fecal samples collected and analysed for this project from Blainville's beaked whales and sperm whales. Samples from 2010 and 2012 were collected opportunistically under funding from other sources.

Year	Beaked Whales	Sperm Whales	Total
2010		3	3
2011	10	9	19
2012		3	3
2013	9	3	12
2014	1	16	17
2015	8	7	15
TOTAL	**28**	**41**	**69**

An additional 18 fecal samples from Blainville's beaked whales (n = 4) and sperm whales (n = 14) collected between 2001-2010 and archived at Woods Hole Oceanographic Institution were processed and assayed for all five hormones (total assayed samples = 87). However, small sample masses and lack of information about sample handling and previous processing rendered these results mostly unreliable with the exception of two sperm whale samples and one beaked whale sample that were included in data analyses.

Information on the age-class, sex and reproductive status based on individual life histories for identified whales is included in Tables 2 and 3. The majority of beaked whale samples were linked to identified whales (n = 23), but most of the subadults were of unknown sex and were analyzed as a single group. Sperm whale samples (of adequate mass-see below) were matched to individuals in the photo-identification catalogue, where possible (n = 20). Six samples were collected from a subadult male sperm whale (Pm156) over a one month, and these samples were averaged for analyses. Therefore, the sample size for statistics was 15 identified sperm whales (Table 3). The age-class of sampled sperm whales was estimated by body size and morphology in some cases without individual identification.

Fecal Hormone assays

Validation studies were successful for RIAs for all five hormones in both species (details in Rolland et al. 2012). The same assay validations were conducted on seawater samples collected in the vicinity of whales. In total, including both species and water samples, 33 different assay validation tests were done for this study. The E1G enzyme immunoassay was also successfully validated, but we elected to use the 125I total estrogens RIA as it was more sensitive, measuring on average 3.3 times higher hormone levels. Control seawater samples (n =13) had non-detectable or extremely low levels of immune-reactivity in all hormone assays that did not significantly alter fecal hormone concentrations.

Beaked Whale Fecal Hormones

Fecal samples from beaked whales with measurable levels of four or more hormones were included in data analyses (n = 24). Mean sample mass was 0.065 ± 0.007g, well above the threshold of 0.02g to avoid effects of low sample mass on hormone concentration (Hayward et al. 2010). Results showed some variability in reproductive hormones based on sex and life history stage (Table 2). For example, adult males had significantly higher testosterone than adult/lactating females and calves ($F_{4,17} = 7.216$, p = 0.001). Total estrogens tended to be lower in subadults, lactating females had much lower progestins compared to other adult females, and calves had lower mean concentrations of most hormones, although these relationships did not reach significance. Finally, the highest progestin value (434.4 ng/g) in an unidentified whale was significantly higher than in all other samples ($t = -11.349$, df = 22, p < 0.001), suggesting that this level may indicate estrus cyclicity (luteal phase) or pregnancy. Therefore, although based on small sample sizes, the beaked whale reproductive hormones showed expected patterns providing biological validation of this approach.

Fecal glucocorticoids (GCs) tended to be higher in adult males and subadults (although not significant), and levels in females and calves were similar (Table 2; Fig. 4). A positive correlation between fecal androgens and GCs in adult males has been reported in other cetaceans (Hunt et al. 2006). Fecal GCs and T3 were significantly correlated (Fig. 5; Pearson $r = 0.433$, p = 0.039). One adult male and an adult female had highly elevated T3 levels (143.3 ng/g and 150.8 ng/g, respectively) compared to other whales, and also had the highest total estrogens in the dataset (180.3 ng/g and 121.5 ng/g). These outlying values for thyroid led to apparent higher mean thyroid hormone in both groups because of the small sample sizes, and also contributed to a positive correlation between fecal estrogens and T3 ($r = 0.750$, p = 0.001).

Table 2. Fecal hormone concentrations (ng/g) for beaked whales by sex, age-class and reproductive state (mean ± SEM) with (median) values below. Adult females are unaccompanied by calves. Superscript numbers indicate sample size differences, and letters indicate significantly different values ([1] n = 4; [2] n = 2).

Age-class/sex	Progestins	Estrogens	Androgens	Glucocort.	Thyroid
Adult female	143.0 ± 94.0	56.9 ± 31.6	4.5 ± 1.6[1]	16.5 ± 4.1	41.7 ± 25.6[1]
(n = 5)	(47.8)	(34.8)	(3.0)	(12.0)	(19.5)
Lactating female	54.7 ± 10.9	50.6 ± 23.1	5.3 ± 1.0	19.9 ± 5.4	27.3 ± 9.1
(n = 5)	(54.9)	(41.8)	(5.2)	(18.4)	(16.2)
Adult male	153.6 ± 26.0	57.1 ± 33.8	63.0 ± 37.1[a]	29.1 ± 5.2	81.2 ± 69.6[2]
(n = 3)	(72.1)	(42.5)	(51.0)	(32.8)	(81.2)
Subadult	105.7 ± 68.4	30.0 ± 11.4	9.3 ± 3.6[a]	33.3 ± 16.2	46.9 ± 20.4
(n = 4)	(39.2)	(27.6)	(6.4)	(21.6)	(29.5)
Calves	48.9 ± 9.4	43.5 ± 10.6	2.5 ± 0.4	15.9 ± 2.1	39.5 ± 12.7
(n = 6)	(43.5)	56.6)	(2.2)	(15.6)	(29.0)
Unknown	434.4[b]	56.5	3.4	12.8	34.1
(n = 1)					

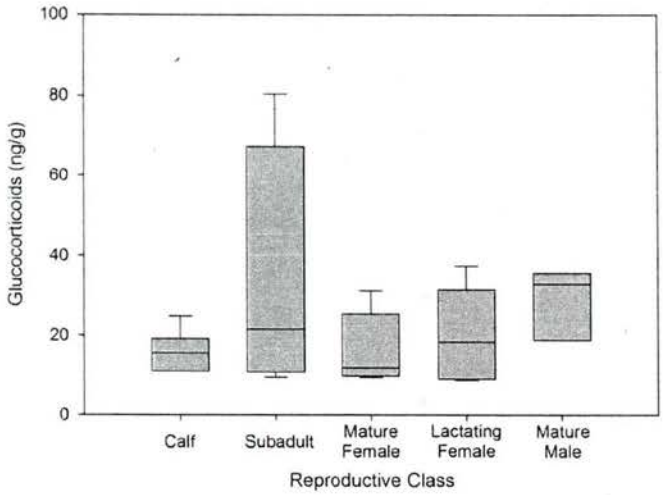

Figure 4. Fecal glucocorticoid concentrations in Blainville's beaked whales by age-class and sex.

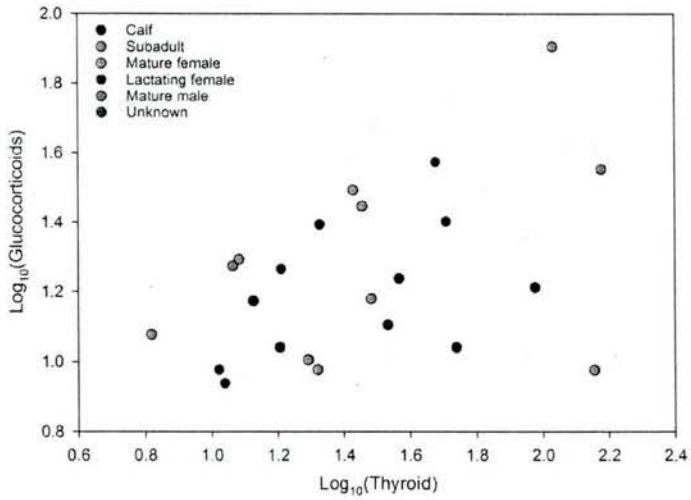

Figure 5. A positive correlation was found between fecal glucocorticoids and thyroid hormones (T3) in Blainville's beaked whales.

Sperm Whale Fecal Hormones

Sperm whale samples > 0.0582g dry weight (1 SD below mean sample dry weight of 0.0817g) were used in analyses for consistency of mass (n = 26), and produced results for all five hormones. Of these, 15 samples were from identified whales and these hormone results were summarized according to age-class (Table 3). Results are presented by age-class instead of further classification by sex because all adult whales were females (with no samples collected from adult males), and many of the subadults were of unknown sex. The "Unknown" category is likely a mix of adults and subadults. One subadult male sperm whale (Pm156) was repeatedly sampled (n = 6) over a one month period. The mean coefficient of variation (CV) for all five hormones in this whale was 11% (range 7-14%). Adult females had both higher progestin and estrogen levels compared to subadults, although there were no statistically significant differences between age-classes for any hormones. Fecal progestins were present at higher concentrations than other hormones in all whales. One adult female and two unknown

whales had highly elevated fecal progestins (> 24,000 ng/g), which is possible evidence of estrus cycling (luteal phase) or pregnancy in the identified adult female, and suggesting that the two unknown samples were likely from adult females. In contrast to beaked whales, sperm whale thyroid hormones had a relatively tight range of values (range = 3.7- 30.3 ng/g) compared to GCs (range = 26.6 - 111.3 ng/g). Thyroid hormone and GCs were not significantly different between age-classes. Further clarification of baseline hormone ranges from sperm whales will require more samples from identified whales, particularly adult males.

Table 3. Fecal hormone concentrations (ng/g) for sperm whales by age-class (mean ± SEM; median values are below). All "Adults" are females.

Age-class	Progestins	Estrogens	Androgens	Glucocort.	Thyroid
Adult	9,504.8 ± 2,513.6	203.9 ± 23.0	100.7 ± 19.5	62.6 ± 4.6	14.1 ± 1.4
(n=8)	(7,757.60)	(190.5)	(85.8)	(64.0)	(15.2)
Subadult	7,174.4 ± 1,523.2	170.1 ±14.5	110.2 ±18.3	58.4 ±5.8	12.56 ± 1.9
(n=12)	(7,026.7)	(178.2)	(109.8)	(57.5)	(10.8)
Unknown	15,695.4 ± 3,471.9	297.2 ± 70.9	94.7 ±22.5	67.5 ± 11.0	11.2 ± 2.4
(n=6)	(13,320.7)	(246.3)	(85.7)	(62.2)	(9.4)

Significant positive correlations between hormones in sperm whales included: 1) progestins and total estrogens (r = 0.610, p = 0.001), 2) androgens and thyroid hormone (r = 0.466, p = 0.016), 3) GCs and progestins (r = 0.539, p = 0.005), and 4) GCs and total estrogens (r = 0.656, p < 0.001). The relationship between GCs and reproductive hormones in sperm whales (Fig. 5) demonstrates the importance of consideration of sex and reproductive status (i.e. other physiologic influences) when interpreting levels of GCs as indicators of stress responses.

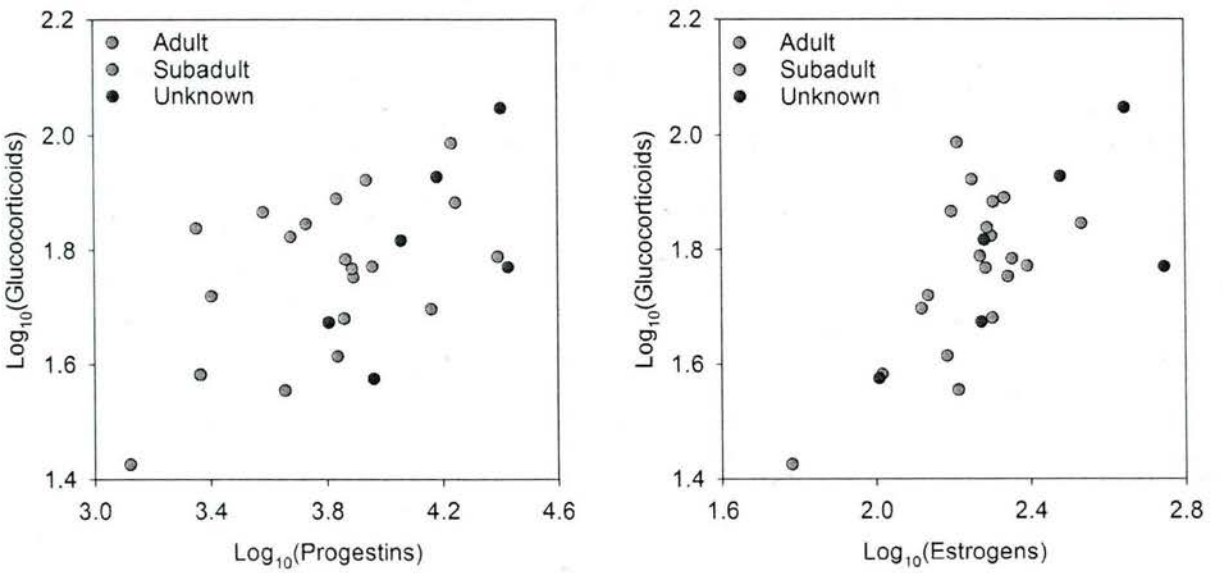

Figure 6. Positive correlations were found between fecal glucocorticoids (GCs) and progestins and total estrogens in sperm whales whales.

IMPACT/APPLICATIONS

Developing methods to better understand the sub-lethal, physiologic consequences of underwater noise disturbance on species of concern, like beaked whales, is crucial to evaluating the potential for long-term impacts of naval exercises and other oceanic activities. The results of this research project have shown that it is feasible to collect fecal samples from both target species, and immunoassays for five classes of fecal hormones were successfully validated in samples from both beaked and sperm whales. These results represent the first hormone data generated on free-swimming beaked and sperm whales, and, although based on small sample sizes, hormone concentrations showed some expected patterns with varying sex and age–class providing evidence of the biological validity of this approach. In summary, this study has developed a physiologic approach to measure stress-related hormones in reference populations of beaked and sperm whales that can be applied in future studies to assess responses of these two species to elevated acoustic exposures from naval activities at the nearby AUTEC range.

RELATED PROJECTS

The NEAq's Marine Endocrinology Program includes a related ONR-funded project on *Development of Novel Noninvasive Methods of Stress Assessment in Baleen Whales* (K. Hunt, PI; ONR #N000141310639). This research project is developing the use of fecal aldosterone assays as an additional measure of adrenal activation during stress responses in North Atlantic right whales (*Eubalaena glacialis*), and is exploring the feasibility of measuring stress-related hormones in respiratory vapor from the same species.

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Rolland RM, Claridge DE, Hunt KE, Dunn CA, Kraus SD. 2013. Assessing Stress Responses in Beaked and Sperm Whales in the Bahamas. Annual Report to the Office of Naval Research for Award Number: N000141110540.

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REPORT DOCUMENTATION PAGE

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DISTRIBUTION STATEMENT A. Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of this project was to develop a non-invasive approach to measure stress-related fecal hormones in minimally disturbed populations of Blainville's beaked whales and sperm whales in the Bahamas that can be applied to assess responses of these two species to known acoustic disturbances. The results showed that fecal sample collection is feasible, immunoassays for five hormones were successfully validated, and varying hormone patterns with different sex and age–class is evidence of the biological validity of this approach. These data can be used in future studies to determine whether acoustic disturbances (including MFA sonar) are causing biologically significant effects on these whale species.

15. SUBJECT TERMS
beaked whale, sperm whale, Bahamas, stress response, fecal hormones, glucocorticoids, thyroid hormone

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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INSTRUCTIONS FOR COMPLETING SF 298

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2. REPORT TYPE. State the type of report, such as final, technical, interim, memorandum, master's thesis, progress, quarterly, research, special, group study, etc.

3. DATE COVERED. Indicate the time during which the work was performed and the report was written, e.g., Jun 1997 - Jun 1998; 1-10 Jun 1996; May - Nov 1998; Nov 1998.

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